Published in final edited form as:

Toxicol Mech Methods. 2017 March ; 27(3): 223–235. doi:10.1080/15376516.2016.1275906.

Effects of Pentachlorophenol and Dichlorodiphenyltrichloroethane on Secretion of Interferon gamma (IFNγ) and Tumor Necrosis Factor alpha (TNFα) from Human Immune Cells

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Abstract

Pentachlorophenol (PCP) and dichlorodiphenyltrichloroethane (DDT) **are pesticides that** have been widely used and significantly contaminate the environment. Both are found in human blood and have been shown to alter the lytic and binding function of human natural killer (NK) cells. Interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) are pro-inflammatory cytokines which regulate immune responsiveness to pathogens and tumors. Their levels require very tight control to prevent loss of immune competence or excessive inflammation. Here we examined the capacity of PCP and DDT to alter the secretion of these critical pro-inflammatory cytokines from increasingly reconstituted (**more complex**) preparations of human immune cells which included NK cells, monocyte-depleted (MD) peripheral blood mononuclear cells (PBMCs) **(a preparation that is predominantly lymphocytes)**, and PBMCs (**a preparation containing lymphocytes and monocytes**). Results indicated that exposure to PCP decreased IFNγ secretion at the highest exposures (2.5 and 5 μM) and increased IFNγ secretion at lower concentrations. These effects were seen irrespective of the complexity of the cell preparation. PCP at 2.5 and 5 μM generally decreased TNFα secretion from NK cells but had inconsistent effects in MD-PBMCs and PBMCs. Exposure of the each of the immune cell preparations to DDT caused increases in IFNγ secretion. **DDT (2.5 μM) increased TNFα secretion from MD-PBMCs after either 24 h or 48 h of exposure**. The mechanism of PCP-induced increases in IFNγ secretion appears to involve the p38 mitogen activated protein kinase (MAPK) pathway, based on loss of PCP stimulated increases when this pathway was inhibited.

Keywords

lymphocytes; monocytes; Pentachlorophenol; Dichlorodiphenyltrichloroethane; Interferon gamma; Tumor necrosis factor alpha

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Declaration of Interest

The Authors report no conflicts of interest. The Authors are alone responsible for the content and writing of the paper.

INTRODUCTION

Pentachlorophenol (PCP) and dichlorodiphenyltrichloroethane (DDT) are organochlorine compounds that contaminate the environment due to their various uses (ATSDR, 2001 ATSDR, 2002; Cirelli, 1978; CDC 2003; 2005; Geisz et al., 2008; Turusov et al., 2002). PCP has been used as a fungicide, insecticide, herbicide, and as a wood preservative (Cirelli, 1978) and is found in human blood at concentrations averaging 0.15 μM in individuals with no known exposure (Cline et al., 1989; Uhl et al., 1986). It is found in much higher levels (0.26 −5 μM) in the serum of those who have lived in log homes where the wood was treated with PCP (Cline et al., 1989). Exposure to PCP has been associated with lymphoma, myeloma and kidney cancer (Demers et al., 2006; Cooper & Jones, 2008). DDT has been widely used as an insecticide and agricultural pesticide (CDC 2003; 2005; Geisz et al., 2008; Turusov et al., 2002). It is no longer used in the USA. However, it is still used to control mosquitos and other pests in many other countries (CDC 2003; 2005) and is found in measurable concentrations in blood samples within the US population (Thornton et al., 2002; Patterson et al., 2009). In those countries where DDT is still used, such as Mexico, it has been found in human serum at concentrations as high as 23,169 ng/g of lipid (approximately 260 nM) (Koepke et al., 2004; Trejo-Acevedo et al., 2009). DDT levels in blood have been associated with decreased numbers of natural killer (NK) cells as well as decreased NK function (Svensson et al., 1994; Eskenazi et al., 2009). Additionally, exposure to DDT has been linked to increased incidences of liver, pancreatic, breast, and testicular cancers as well as leukemia (Eskanazi et al., 2009; Cohn et al., 2007)

The pro-inflammatory cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) regulate the functions of both innate and adaptive immune cells. IFNγ increases antigen presentation on macrophages by increasing their expression of MHC class I molecules and regulates T cell immune response through Th1 cells. It is also involved in the recruitment of innate immune cells to sites of infection or tumor (Zaidi & Merlino, 2011). IFNγ is secreted by T cells, NK cells, and to a lesser extent by myeloid lineage cells such as macrophages (Billiau & Matthys, 2009; Darwich et al., 2008). It is an approximately 17kD protein and is dimerized in its physiologically active form (Billau & Matthys, 2009). TNFα is initially produced as a 26 kD transmembrane protein that is then released from the membrane as a 17kD protein by a wide array of cells including T cells, natural killer (NK) cells, and monocytes (Goetz et al., 2004). In addition to activating the inflammatory immune response, it is also able to causes apoptosis as well as cell proliferation (Guicciardi & Gores, 2009; Silke, 2011). Both IFNγ and TNFα are a potent inflammatory stimuli and as such have the capacity to cause chronic inflammation. Chronic inflammation has been shown to be associated with a number of disease states including, rheumatoid arthritis, Crohn's disease and several cancers (Macarthur et al., 2004; Tracey et al., 2008; Grivennikov & Karin, 2011). Thus, it important that IFNγ and TNFα levels in the body are carefully regulated to prevent either a loss of immune competency or the risks that occur due to chronic inflammation.

Previous studies have shown that the ability of human NK cells to destroy tumor target cells is inhibited by both PCP and DDT. Additionally, both of these compounds decreased the ability of NK cells to bind to tumor target cells (Reed et al., 2004; Nnodu & Whalen, 2008;

Udoji et al., 2010; Hurd et al., 2012; Hurd-Brown et al., 2013) while decreasing the expression of certain cell-surface proteins needed for the binding process (Hurd et al., 2012; Hurd-Brown et al., 2013). Based on the results from previous studies on the effects of PCP and DDT on immune function we hypothesize that PCP and DDT may alter the secretion of IFNγ and/or TNFα from human immune cells.

The current study examined the effects of exposures to PCP or DDT exposures on secretion of IFNγ and TNFα from NK cells, monocyte-depleted (MD) peripheral blood mononuclear cells (PBMCs), and PBMCs. Examination of the effects of these compound on secretion of these cytokines from preparations of immune cells t**hat contained different cell types** allowed us to determine whether the effects of the compounds vary as the system becomes more reconstituted **(NK lymphocytes versus T and NK lymphocytes (MD-PBMCs) versus T and NK lymphocytes + monocytes (PBMCs))** and thus more closely resembles the physiological setting. An additional goal of this study was to investigate the mechanism of any increases in cytokines induced by exposure to the compounds, using specific signaling pathway inhibitors.

MATERIALS AND METHODS

Preparation of NK cells

NK cells were prepared from buffy coats (source leukocytes from healthy adult donors) purchased from Key Biologics, LLC (Memphis, TN) or leukocyte filters (PALL-RCPL or FLEX) obtained from the Red Cross Blood Bank (Nashville, TN) using a rosetting procedure. RosetteSep human NK cell enrichment antibody cocktail (0.6–0.8 mL) (StemCell Technologies, Vancouver, British Columbia, Canada) was added to 45 mL of buffy coat (as decribed by the manufacturer) (Aoukaty & Tan, 2005; Almughamsi & Whalen, 2016). The mixture was incubated for 20 min at room temperature (~ 25° C). Approximately 8 mL of the mixture was layered onto 4 mL of LymphoSep™ cell separation medium (1.077 g/mL) (MP Biomedicals, Irvine, CA) and centrifuged at 1200 g for 30–50 min. NK cells were collected and washed twice with phosphate buffered saline (PBS) pH 7.4 and stored in complete media (RPMI-1640 supplemented with 10% heat-inactivated bovine calf serum (BCS), 2 mM L-glutamine and 50 U penicillin G with 50 μg streptomycin/ml) at 1 million cells/mL at 37 °C and air/CO_2, 19:1.

Preparation of monocyte- depleted PBMCs and PBMCs

PBMCs were isolated from Leukocyte filters (PALL- RC2D) obtained from the Red Cross Blood Bank Facility (Nashville, TN) as described in Meyer et al., 2005. **Filters are used by the Red Cross to remove white blood cells from units of blood. Each filter represents a separate blood donor and is numbered.** Leukocytes were retrieved from the filters by back-flushing them with elution medium (PBS containing 5 mM disodium EDTA and 2.5% [w/v] sucrose) and collecting the eluent. Eluent contained the leukocytes with red cell contamination. Eluent was layered onto LymphoSep™ (1.077g/mL) and centrifuged as described above. Granulocytes and red cells pelleted at the bottom of the tube while the PBMCs floated on the LymphoSep™. PBMCs were collected and washed (250 g, 10 min.) with PBS. Cells were then suspended in complete medium which consisted of RPMI-1640

supplemented with 10% heat-inactivated BCS, 2 m*M L*-glutamine and 50 U penicillin G with 50 μg streptomycin/mL. This preparation constituted PBMCs. **Monocyte-depleted PBMCs (10–20% $CD16^+$, 10–20 % $CD56^+$, 70–80% $CD3^+$, 3–5% $CD19^+$, 2–20% $CD14^+$) were prepared by incubating PBMCs in glass Petri dishes (150 × 15 mm) at 37 °C and air/CO_2, 19:1 for 45 min. The cell suspension was then removed and placed onto clean glass Petri dishes for another 45 min at 37 °C and air/CO_2, 19:1. The non-adherent cells were then collected from the petri dishes. The resulting preparation was predominantly NK and T lymphocytes as the monocytes and B lymphocytes adhered to the glass** (Koller et al., 1973).

Chemical Preparation

DDT and PCP were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared as 100 mM solutions in Dimethylsulfoxide (DMSO). Desired concentrations of either DDT or PCP were prepared by dilution of the desired stock into cell culture media. **Appropriate DMSO dilutions were used as controls. DMSO was never greater than 0.02% in any of the incubations.**

Inhibitor Preparation

Enzyme inhibitors were purchased from Fischer Scientific (Pittsburgh, PA). The stock solution for each inhibitor was a 50 mM solution in dimethylsulfoxide (DMSO). MEK 1/2 pathway inhibitor (PD98059), p38 inhibitor (SB202190), JNK inhibitor(B178D3), and NFκB inhibitor (BAY11-7085) were prepared by dilution of the stock solution into cell culture media mixture.

Cell Treatments

PBMCs, MD-PBMCs, and NK cells (at a concentration of 1.5 million cells/ mL) were treated with PCP (0.05–5 μM) or DDT (0.025–2.5 μM) and appropriate control for 24 h, 48 h, and 6 days. Once the incubation period was complete, the cells were pelleted and supernatants were obtained and stored at −70 C until assaying for IFNγ or TNFα. For the pathway inhibitor experiments, PBMCs were treated with enzyme inhibitors 1 h prior to adding PCP at concentrations of 0.25, 0.1, and 0.05 μM PCP for 24 h. After the cells were incubated, the cells were pelleted and supernatants were obtained and stored at −70 C until assaying for IL-1β.

Cell Viability

Cell viability was measured at the end of the 24 h, 48 h and 6 day exposure period. Viability was determined using the trypan blue exclusion method. Cells were mixed with trypan blue and counted using a hemocytometer. The total number of cells and the number of dead were counted for both control and treated cells to determine the percent viable cells.

IFNγ and TNFα secretion assays

IFNγ or TNFα levels were assessed using OptEIA™ enzyme-linked immunosorbent assay (ELISA) human IFNγ or TNFα kit (BD-Pharmingen, San Diego, CA). A 96 well microwell plate specific to ELISA was incubated with Capture antibody overnight. The capture

antibody was removed by washing with PBS containing 0.05% Tween-20. Non-specific binding was blocked with by incubation with blocking buffer for 1 h. This incubation was followed by removal of the blocking solution and incubation of the plate with cell supernatants and IFNγ or TNFα standards for 2 h. Following the incubation with samples and standards they were removed and detection antibody was added and incubated for 1 h. Removal of detection antibody was followed by application of the substrate solution for a 30 min incubation. Incubation with substrate was ended by addition of acid and the absorbance was measured at 450 nm on a Thermo Labsystems Multiskan MCC/340 plate reader (Fisher Scientific).

Statistical analysis

Statistical analysis of the data was carried out utilizing ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data using Student's t test, a p value of less than 0.05 was considered significant.

RESULTS

Viability of NK cells, MD-PBMCs, and PBMCs exposed to PCP and DDT

When immune cells were exposed to concentrations of PCP from 0.05 to 5 μM, there was no effect on the viability of the cells compared to control cells. Data not shown. The viability of NK cells, MD-PBMCs, and PBMCs was also unaffected by exposure to DDT. Data not shown.

Viability of monocyte-depleted PBMCs treated with selective enzyme inhibitors and then exposed to PCP

The effects of exposures to selective enzyme inhibitors in the presence and absence of 0.05,0,1, 0.25 μM PCP on the viability of MD-PBMCs was also examined. The presence of inhibitors did not negatively affect the viability of the MD-PBMCs compared to control cells.

Secretion of IFNγ and TNFα from NK cells exposed to PCP

Effects of PCP exposures on IFNγ secretion are shown in Table 1A. IFNγ secretion was increased by exposure to 1 or more concentrations of PCP after 24 h. The concentrations that produced increased secretion varied from donor to donor. However, 0.1 μM PCP caused a significant increase in IFNγ secretion in all donors tested. The magnitude of the increase in secretion following a 24 h exposure to PCP also varied among donors. The range for maximum fold increase in IFNγ secretion stimulated by PCP was 1.5 fold (F345) to 8.8 fold (F338). PCP-induced increases in IFNγ secretion (at 1 or more concentration) were also seen at the 48 h and 6 day exposures. In addition to the PCP-stimulated increases, there were also PCP-stimulated decreases in IFNγ after 24 h exposures to 2.5 and 5 μM PCP in cells from each of the donors (Table 1A). This pattern maintained at 48 h and 6 days. Figure 1A shows data from a representative experiment (F365). There were significant decreases in TNFα secretion from 3 of 4 donors at 24 hrs. However the extent of the decrease varied

from donor to donor (Table 1B). This same pattern of effect on TNFα secretion was seen after 48 h and 6 days of exposure to PCP.

Secretion of IFNγ and TNFα from MD-PBMCs exposed to PCP

As with the NK cells, MD-PBMCs (a preparation is largely NK cells and T cells) were exposed to PCP (0.05–5 μM) for 24 h, 48 h, and 6 d after which IFNγ secretion was measured (Table 2A). Of the 5 donors that were studied, cells from 4 donors showed statistically significant increases in IFNγ secretion with at least 1 concentration of PCP after a 24 h exposure. Cells from all donors showed an increase in secretion after 48 h exposures to PCP at one or more concentration. The maximum fold increase seen after 48 h ranged from 1.3 fold (F209) to 20.5 fold (F314). Similar results were seen after 6 d of exposure to PCP. **Results from a representative experiment (F312) are in**Figure 1B. The effects of exposing MD-PBMCs to PCP on TNFα secretion are shown in Table 2B. There was no consistent effect of any given concentration of PCP on secretion of TNFα from cells from any of the donors at any length of incubation.

Secretion of IFNγ and TNFα from PBMCs exposed to PCP

PBMCs (a cell preparation containing lymphocytes and monocytes) were exposed to PCP as described above (Table 3A). PBMCs from all donors showed a statistically significant increase in IFNγ when exposed to one or more concentration of PCP. The concentrations at which significant increases were seen varied among the donors tested as did the length of incubation at which the increase occurred. Cells from 3 of 4 donors showed increases in IFNγ secretion at the 24 h exposure. These increase ranged from 2.3 (F197) to 5.1 (F214). The fourth donor (F238) showed a significant increase in IFNγ secretion after 6 days of exposure. All donors showed significant decreases in IFNγ secretion at either the 5 or 2.5 μM concentrations of PCP at each length of exposure. Figure 1 C shows the data from F211. PBMCs did not show any consistent alterations of TNFα secretion when exposed to PCP (Table 3B).

Secretion of IFNγ and TNFα from NK cells exposed to DDT

NK cells were exposed to 0.025 to2.5 μM DDT and appropriate control for 24 h, 48 h, and 6 d and IFNγ secretion was measured (Table 4A). As was seen with PCP exposures, there were statistically significant increases in IFNγ secretion with exposure to at least one concentration of DDT at one or more length of exposure. Cells from 3 of 4 donors showed statistically significant increases after 24 h of exposure while cells from donor F361 showed an increase in IFNγ secretion by the 48 h time point. Concentrations that caused increases varied from one donor to the next as did the magnitude of the increase. The maximum fold increases after 24 h exposures to DDT ranged from 1.5 fold (F375) to 3.6 fold (F366). Figure 2A shows representative results from F366. **Similar effects were seen in most donors after 48 h. DDT-induced increases tended to diminish in cells exposed for 6 days possibly due to the length of time in culture.** Exposure of pure NK cells to 0.025–2.5 μM DDT had little effect on the secretion of TNFα as compared to the control. This was the case in cells from all donors at all lengths of exposure (Table 4B).

Secretion of IFNγ and TNFα from MD-PBMCs exposed to DDT

There were significant increases in IFNγ secretion from MD-PBMCs of all donors within 48 h of exposure to DDT (Table 5A). The specific concentration that caused a significant increase differed among donors as did the maximum fold increase. For instance, cells from F308 showed a 1.4 fold increase in IFNγ secretion at the 1 μM exposure after 48 h, while cells from F304 exhibited an 8.8 fold increase at the 0.05 μM concentration of DDT after the same length of exposure. Representative results from F308 are shown in Figure 2B. **As was seen with NK cells, increases in IFNγ seen with DDT exposures tended to decrease or disappear after 6 days of exposure.** Increases in TNFα secretion were seen at the higher concentrations of DDT in cells from all donors at one or more length of exposure (Table 5B). The increases were 1.64, 1.33, and 1.42 fold as compared to the control in F106, F178, and F180 at 24 h and 1.78 fold for F174 at 48 h. Results from a representative experiment (F106) are shown in Figure 2C.

Secretion of IFNγ and TNFα from PBMCs exposed to DDT

PBMCs from all donors showed significant increases in IFNγ secretion at a minimum of one concentration of DDT over the course of the 6 day exposures (Table 6A). Figure 2D shows the results from a representative experiment (F201). Exposure of PBMCs to DDT for 24 h, 48 h, and 6 d had variable effects on secretion of TNFα (Table 6B).

PCP-induced Secretion of IFNγ from MD-PBMCs pre-treated with Selective Enzyme Inhibitors

NFκB Inhibitor (BAY 11-7085)—The effect of NFκB inhibition on PCP-induced increases in IFNγ were examined at 0.05, 0.1, and 0.25 μM PCP after a 24 h incubation. These concentrations were chosen based on the fact that, in the studies presented above, they were able to induce an increase in IFNγ secretion from the MD-PBMCs of 4 of the 5 donors. NFκB was inhibited by pre-treating the MD-PBMCs with BAY 11-7085 (0.325 μM) for 1 h prior to a 24 h exposure to PCP. Inhibition of NFκB did not prevent PCP from stimulating an increase in IFNγ secretion from MD-PBMCs (Table 7). For example, there is a 2 fold increase in IFNγ secretion when MD-PBMCs from donor F374 are exposed to 0.25 μM PCP in the absence of NFκB inhibitor. When the inhibitor is present this same PCP exposure causes a 3 fold increase in IFNγ secretion (Figure 3A). These results indicate that NFκB is not utilized by PCP to stimulate IFNγ secretion.

Mitogen activated protein kinase (MAP2K), MEK, Inhibitor (PD98059)—PCP-stimulated secretion of IFNγ from MD-PBMCs where MEK had been inhibited with PD98059 (50 μM) was also examined (Table 7). Cells from 6 of the 8 donors exposed to PCP showed a lower fold increase in IFNγ secretion in the presence of the inhibitor. There was a 1.6 fold increase in IFNγ secretion from cells from donor F326 when MEK was active and no increase when MEK was inhibited (Figure 3B). These results suggest that the p44/42 pathway may be being utilized by PCP to stimulate IFNγ secretion from MD-PBMCs from most donors.

p38 Inhibitor (SB202190)—Table 7 shows the effects of exposures to PCP on secretion of IFNγ from MD-PBMCs in the presence and absence of the p38 inhibitor SB202190.

Inhibition of p38 decreased the PCP-induced increases in IFNγ secretion in cells from each of the 4 donors tested. MD-PBMCs from donor F331 demonstrated a 1.7 fold increase when exposed to 0.1 μM PCP in the absence of p38 inhibitor and no increase when p38 was inhibited (Figure 3C). These data suggest that PCP is utilizing the p38 pathway to stimulate increases in IFNγ secretion.

JNK Inhibitor (BI78D3)—PCP-stimulated secretion of IFNγ was also examined in MD-PBMCs where JNK had been inhibited with BI78D3 (Table 7). Cells from donors F326, F331, F370, F374, and F385 showed little to no effect of inhibiting JNK on the ability of PCP to stimulate IFNγ secretion. The fold increase in IFNγ secretion in response to 0.25 μM PCP from F370 was 1.6 fold when JNK was not inhibited and 3 fold when JNK was inhibited. Cells from donors F326 showed increased ability of 0.25 μM PCP to stimulate IFNγ secretion when JNK was inhibited (1.4 fold in the absence of inhibitor versus 2.4 fold in the presence of inhibitor) (Figure 3D). These results suggest that the JNK pathway is not utilized to any great extent by PCP to stimulate increases in IFNγ secretion.

DISCUSSION

Due to their uses in insect control, wood preservation and other applications (CDC, 2003; 2005; Cirelli, 1978; U.S. EPA, 2001), DDT and PCP are found in human blood samples (Thornton et al., 2002; Patterson et al., 2009; Cline et al., 1989; Uhl et al., 1986; CDC, 2005). Serum DDT levels as high as 23,169 ng/g of lipid (approximately 260 nM) have been found (Koepke et al., 2004; Trejo-Acevedo et al., 2009). Decreases in NK cell numbers and function have been associated with blood levels of DDT (Eskenazi et al., 2009; Svensson et al., 1994). PCP has been detected at an average level of 0.15 μM for individuals with no known exposure and levels of 0.26 −5 μM in the serum of individuals residing in PCP-treated log homes (Cline et al., 1989; Uhl et al., 1986). Both DDT and PCP are associated with increases in the incidences of certain cancers (Demers et al., 2006; Cohn et al., 2007). Past studies have shown that both PCP and DDT inhibit the lytic function of human NK cells and decrease their expression of key cell surface proteins (Udoji et al., 2010; Reed et al., 2004; Taylor et al., 2005; Nnodu & Whalen, 2008; Hurd et al., 2013).

IFNγ is an important pro-inflammatory cytokine (Billiau & Matthys, 2009; Darwich et al., 2008; Kraaij et al., 2014). It has role in both decreasing the growth of tumors and in some cases enhancing their growth (Zaidi & Merlino, 2011) as well as in controlling infections (Schroder et al., 2004). TNFα elevation is associated with a number of cancers including bladder, breast and prostate (Balkwill & Mantovani, 2001) and can stimulate factors that increase invasiveness and metastasis of cancer cells (Kamata et al., 2005). Elevated levels of TNFα also appear to be part of the pathology of Crohn's disease, psoriasis, and rheumatoid arthritis (Tracey et al., 2008). Thus, dysregulation of IFNγ and TNFα secretion (either too much or too little) could lead to accelerated tumor growth, diseases such as rheumatoid arthritis, or infection (Zaidi & Merlino, 2011; Tracey et al., 2008; Balkwill & Mantovani, 2001). Previous studies have shown that butyltin and brominated flame retardant environmental contaminants are able to disrupt the secretion of IFNγ from immune cells (Lawrence et al., 2015; Almughamsi & Whalen, 2016) and that butyltins are able to alter secretion of TNFα (Hurt et al., 2012). Thus, the current study examined whether DDT

and/or PCP exposures in ex vivo immune cell preparations lead to alterations of IFNγ and /or TNFα secretion. Three immune cell preparations of varying complexity were used in examining the effects of PCP and DDT on secretion of these cytokines, in order to discern whether the composition of the cell preparation influences any effects seen with the compounds.

The results of this study showed that PCP caused both increases and decreases in IFNγ secretion depending on the concentration of PCP to which the cells were exposed. Decreases in IFNγ secretion were seen at either the 2.5 or 5 μM PCP exposures in each of the different cell preparations (NK, ND-PBMCs, PBMCs) after 24 h. The concentration range where increases occurred was at the lower levels of exposure (0.5–0.05 μM). The effects seen after a 24 h exposure to PCP tended to maintain after 48 h and 6 day exposures to PCP in each of the different cell preparations. After 24 h the average maximum fold increase in IFNγ secretion for NK cells was 4.3 fold (range: 1.5–8.8 fold) with all donors showing some increase. The average maximum fold increase in IFNγ secretion seen in MD-PBMCs after a 24 h exposure was 2.4 fold (range: 1.5–4.8 fold) and was seen in 4 of 5 donors and that of the PBMCs was 3.7 fold (range 2.3–5 fold) and was seen in 3 of 4 donors. Thus, there was a relatively broad range of increases in each of the cell preparations that was donor dependent after 24 h of exposure.

Previous studies have shown that exposure to PCP for 24 h lead to decreases in the ability of NK lymphocytes to destroy tumor cells (lytic function) (Reed et al., 2004, Nnodu & Whalen, 2008). Decreases in lytic function were seen at higher PCP concentrations while concentrations below 0.5 μM had no effect on this function. The current study shows that IFNγ secretion is also decreased at the highest PCP exposures in NK lymphocytes. Those concentrations that caused little to no effect on lytic function however, are able to increase the ability of the NK cell to secrete IFNγ. Thus, while exposures to high concentrations of PCP seem to be generally inhibitory to NK cell function, exposures to lower levels stimulate the ability of the NK cell to produce the important immune response activator, IFNγ. As mentioned above, exposure to PCP is associated with increases in the incidences of certain cancers (Demers et al., 2006). However, the concentration of PCP in individuals who developed these cancers was not determined.

The effects of PCP exposure on IFNγ secretion were similar among all the cell types examined (NK cells, MD-PBMCs, and PBMCs). **There tended to be a biphasic effect of PCP on IFNγ secretion in each of the cell preparations. As was noted above, higher concentrations of PCP (5 and 2.5 μM) tended to decrease IFNγ secretion and lower concentrations (0.1 and 0.05 μM) tended to increase its secretion. A possible explanation for this biphasic effect is that at the higher concentration PCP would interact with a very wide spectrum of cellular components and this would result in multiple simultaneous (and possibly competing) effects, with the resulting overall impact on the cell being inhibitory of its function. As the concentration of PCP decreases (such as at 0.1 or 0.05 μM), it will interact with far fewer components and the result of those interaction will be to stimulate the secretion of IFNγ.**

No consistent effects on TNFα secretion were seen when these immune cell preparations were exposed to PCP. This was in contrast to the consistent decreases and increases seen in IFNγ secretion. Thus, the effects of PCP on secretion of these 2 cytokines are markedly different. This may reflect the fact that regulation of their secretion and production while having overlap is distinct for each of these cytokines (Tsukaguchi et al., 1999).

Exposure of the various cell preparations to DDT caused increases in IFNγ secretion at one or more concentration after 48 h of exposure. This effect was seen in each of the different types of cell preparation (NK, MD-PBMCs, PBMCs). The concentrations of DDT where increases in IFNγ secretion were seen ranged from the highest concentration tested (2.5 μM) to the lowest concentration (0.025 μM), this is in contrast to PCP where only the lower concentrations (0.5–0.05 μM) caused increased secretion. Additionally, the most consistent and greatest fold increases seen with DDT occurred after 48 h of exposure while occurring after 24 h with PCP treatments. DDT caused no consistent decreases in IFNγ in any of the cell preparations while PCP consistently caused decreases at the 2.5 and/or 5 μM exposures. DDT exposures increased TNFα secretion from MD-PBMCs after 24 or 48 h. However, there were no consistent effects of DDT exposures in either NK cells or PBMCs.

Previous studies have shown that other compounds that contaminate the environment, brominated flame retardants and butyltins, are able to alter the secretion of IFNγ from immune cells (Almughamsi & Whalen, 2016; Lawrence et al. 2015). The flame retardant, HBCD, and the butyltins, tributyltin (TBT) and dibutyltin (DBT), all tended to increase IFNγ secretion at the lower exposure concentrations, as did PCP. Another pro-inflammatory cytokine, IL-1β has also been shown to be increased by exposures to HBCD and TBT (Anisuzzaman & Whalen, 2016; Brown & Whalen, 2015).

As mentioned above, PCP tended to cause consistent increases in IFNγ secretion from MD-PBMCs at concentration between 0.25 and 0.05 μM. Thus, we examined the role of various signaling pathways in regulating the PCP-induced increases in IFNγ secretion. IFNγ levels in immune cells can be regulated by a variety of signaling pathways including NFκB and the MAPKs, p38, p44/42(also known as ERK1/2) and JNK (Schoenborn & Wilson, 2007; Girart et al., 2007; Samten et al., 2008). The results indicated that both the p44/42 and the p38 MAPK pathways may be stimulated by PCP exposures leading to increased IFNγ secretion. Previous studies indicated that HBCD-induced increases in IFNγ secretion were dependent on the p44/42 pathway as well (Almughamsi & Whalen, 2016). Additionally, increases in IL-1β stimulated by HBCD and TBT were also dependent on the p44/42 pathway.

PCP and DDT showed variations in their effects on IFNγ secretion (specific lengths and concentrations of exposure) that was donor dependent. Indicating that there may be significant differences in the consequences of exposures to these (and other chemical contaminants) from one individual to the next. This would be important in assessing the risk from exposures for a variety of potential health consequences. The concept of personalized treatment of a number of diseases, most especially cancer (Madureira & de Mello, 2014) has begun to evolve. The results from this study indicate that there may individualized responses to these environmental toxicants.

Due to the importance of IFNγ in regulating immune responses to pathogens and its potential to cause unwanted inflammation, the alterations of its secretion from immune cells, as have been seen with PCP and DDT exposures, have significant implications. For instance if levels are decreased, as was seen with 2.5 and 5 μM PCP, then an inadequate stimulation of other components of the immune response would occur. Conversely, if levels are increased by PCP or DDT (as seen in this study), the potential for unwarranted inflammation occurs. Such excessive inflammation is capable of fueling pathological responses such as atherosclerosis as well as tumor growth and development (Tracey et al., 2008; Macarthur et al., 2004). Inflammation occurring in the absence of a microorganism has been referred to as "sterile inflammation" (Chen & Nunez, 2010). Thus, compounds such as PCP or DDT could result in sterile inflammation in exposed individuals due to their ability to induce the production of pro-inflammatory cytokines such as IFNγ.

A previous study showed that butlytins (both TBT and DBT) altered secretion of TNFα from human white blood cells. That study showed that TBT caused decreased secretion of TNFα in NK cells. However TBT (at lower concentrations) stimulated secretion of TNFα in cell preparations that contained both T and NK cells (monocyte-depleted PBMCs). DBT at higher concentrations also decreased TNFα secretion and increased secretion at lower concentrations in both NK cell and the MD-PBMCs (Hurt et al., 2013). This is in contrast to the effects of PCP and DDT on secretion of TNFα where there was no consistent modification of secretion except with PCP exposures in the MD-PBMCs.

In summary, the current study shows that secretion of IFNγ in three distinct immune cell preparations is altered by PCP and DDT exposures. Exposures to PCP result in significant decreases in IFNγ secretion at the highest exposure levels (2.5 and 5 μM) and in increases in IFNγ secretion at lower concentrations. The PCP-induced increases in IFNγ appear to utilize both the p44/42 (ERK1/2) and p38 MAPK pathways. Additionally, DDT stimulates secretion of IFNγ from each of the three cell preparations.

Acknowledgments

Grant U54CA163066 from the National Institutes of Health

References

Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Pentachlorophenol. Atlanta, GA: 2001. Update

Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for DDT, DDE, DDD. Agency for Toxic Substances and Disease Registry; Atlanta, GA: p. 2002

Almughamsi H, Whalen MM. Hexabromocyclododecane and Tetrabromobisphenol A alter secretion of interferon gamma (IFNY) from human immune cells. Archives of Toxicol. 2016; 90:1695–1707.

Aoukaty A, Tan R. Role for glycogen synthase kinase-3 in NK cell cytotoxicity and X-linked lymphoproliferative disease. J Immunol. 2005; 174:4551–4558. [PubMed: 15814676]

Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001; 357:539–545. [PubMed: 11229684]

Billiau A, Matthys P. Interferon-γ: A historical perspective. Cytokine & Growth Factor Reviews. 2009; 20:97–113. [PubMed: 19268625]

Centers for Disease Control and Prevention (CDC). 2nd National Report on Human Exposure to Environmental Chemicals. 2003

Centers for Disease Control and Prevention (CDC). 3rd National Report on Human Exposure to Environmental Chemicals. 2005

Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nature Reviews/ Immunology. 2010; 10:826–837.

Cline RE, Hill RH Jr, Phillips DL, Needham LL. Pentachlorophenol measurements in body fluids of people in log homes and workplaces. Arch Environ Contam Toxicol. 1989; 18:475–481. [PubMed: 2774665]

Cirelli, DP. Patterns of pentachlorophenol usage in the United States of America-an overview. In: Rao, KR., editor. Pentachlorophenol, chemistry, pharmacology, and environmental toxicology. New York, NY: Plenum Press; 1978. p. 13-18.

Cohn BA, Wolff MS, Cirillo PM, Sholtz RI. DDT and breast cancer in young women: new data on the significance of age at exposure. Environ Health Perspect. 2007; 115:406–414.

Cooper GS, Jones S. Pentachlorophenol and Cancer Risk: Focusing the Lens on Specific Chlorophenols and Contaminants. Environmental Health Perspectives. 2008; 116:1001–1108. [PubMed: 18709150]

Darwich L, Coma G, Peña R, Bellido R, Blanco EJ, Este JA, Borras FE, Clotet B, Ruiz L, Rosell A, Andreo F, Parkhouse RM, Bofill M. Secretion of interferon-γ by macrophages demonstrated at the single-cell level after co-stimulation with interleukin (IL)-12 plus IL-18. Immunology. 2008; 126:386–393. [PubMed: 18759749]

Demers PA, Davies HW, Friesen MC, Hertzman C, Ostray A, Hershler R, Teschke K. Cancer and occupational exposure to pentachlorophenol and tetrachlorophenol (Canada). Cancer causes control. 2006; 17:749–758. [PubMed: 16783603]

Eskenazi B, Chevier J, Rosas LG, Anderson HA, Bornman MS, Bouwman H, Chen A, Cohn BA, deJager C, Henshel DS, Leipzig F, Leipzig JS, Lorenz EC, Snedeker SM, Stapleton D. The Pine River Statement: Human Health Consequences of DDT Use. Environ Health Perspec. 2009; 117:1359–1367.

Geisz HN, Dickhut RM, Cochran MA, Fraser WR, Ducklow HW. Melting glaciers: a probable source of DDT to the Antarctic marine ecosystem. Environmental Science & Technology. 2008; 42:3958–3962. [PubMed: 18589951]

Girart MV, Fuertes MB, Domaica CI, Rossi LE, Zwirner NW. Engagement of TLR3, TLR7, and NKG2D regulate IFN-γ secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. J Immunol. 2007; 179:3472–3479. [PubMed: 17804388]

Goetz FW, Planas JV, MacKenzie S. Tumor necrosis factors. Developmental and Comparative Immunology. 2004; 28:487–497. [PubMed: 15062645]

Grivennikov SI, Karin M. Inflammatory cytokines in cancer: tumor necrosis factor and interleukin 6 take the stage. Ann Rheum Dis. 2011; 70(Suppl. 1):i104–i108. [PubMed: 21339211]

Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009; 23:1625–1637. [PubMed: 19141537]

Hurd-Brown T, Udoji F, Martin T, Whalen MM. Effects of DDT and Triclosan on Tumor-cell Binding Capacity and Cell-Surface Protein Expression of Human Natural Killer Cells. Journal of Appl Toxicol. 2013; 33:495–502. [PubMed: 22729613]

Hurd T, Walker J, Whalen MM. Pentachlorophenol decreases tumor-cell-binding capacity and cell-surface protein expression of human natural killer cells. J Appl Toxicol. 2012; 32:627–634. [PubMed: 22912977]

Hurt K, Hurd-Brown T, Whalen M. Tributyltin and dibutyltin alter secretion of tumor necrosis factor alpha from human natural killer cells and a mixture of T cells and natural killer cells. Journal of Applied Toxicology. 2013; 33:503–510. [PubMed: 23047847]

Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF alpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell. 2005; 120:649–661. [PubMed: 15766528]

Koepke R, Warner M, Petreas M, Cabria A, Danis R, Hernandez-Avila M, Eskenazi B. Serum DDT and DDE levels in pregnant women of Chiapas. Mexico Arch Environ Health. 2004; 59:559–565. [PubMed: 16599003]

Koller CA, King GW, Hurtubise PE, Sagone AL, LoBuglio AF. Characterization of glass adherent human mononuclear cells. J Immunol. 1973; 111:1610–1612. [PubMed: 4583079]

Kraaij MD, Vereyken EJ, Leenen PJ, van den Bosch TP, Rezaee F, Betjes MG, Baan CC, Rowshani AT. Human monocytes produce interferon-gamma upon stimulation with LPS. Cytokine. 2014; 67:7–12. [PubMed: 24680476]

Lawrence S, Reid J, Whalen M. Secretion of interferon gamma from human immune cells is altered by exposure to tributyltin and dibutyltin. Environmental Toxicology. 2015; 30:559–571. [PubMed: 24357260]

Macarthur M, Hold GL, El-Omar EM. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. American Journal of Physiology Gastrointestinal and Liver Physiology. 2004; 286:G515–G520. [PubMed: 15010360]

Meyer TP, Zehnter I, Hofmann B, Zaisserer J, Burkhart J, Rapp S, Weinauer F, Schmitz J, Illert WE. Filter Buffy Coats (FBC): A source of peripheral blood leukocytes recovered from leukocyte depletion filters. Journal of Immunological Methods. 2005; 307:150–166. [PubMed: 16325197]

Nnodu U, Whalen MM. Pentachlorophenol decreases ATP levels in human natural killer cells. J Appl Toxicol. 2008; 28:1016–1020. [PubMed: 18623605]

Patterson DG Jr, Wong LY, Turner WE, Caudill SP, Dipietro ES, McClure PC, Cash TP, Osterloh JD, Pirkle JL, Sampson EJ, Needham LL. Levels in the U. S. population of those persistent organic pollutants (2203–2004) included in the Stockholm Convention or in other long-range trans-boundary air pollution agreements. Environ Sci Technol. 2009; 43:1211–1218. [PubMed: 19320182]

Reed A, Dzon L, Loganathan BG, Whalen MM. Immunomodulation of human natural killer cell cytotoxic function by organochlorine pesticides. Human Exp Toxicol. 2004; 23:463–471.

Samten B, Townsend JC, Weis SE, Bhoumik A, Klucar P, Shams H, Barnes PF. CREB, ATF, and AP-1 transcription factors regulate IFN-γ secretion by human T cells in response to mycobacterial antigen. Journal of Immunol. 2008; 181:2056–2064. [PubMed: 18641343]

Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology. 2004; 75:163–189. [PubMed: 14525967]

Schoenborn JR, Wilson CB. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. Adv Immunol. 2007; 96:41–101. [PubMed: 17981204]

Silke J. The regulation of TNF signalling: what a tangled web we weave. Curr Opin Immunol. 2011; 23:620–626. [PubMed: 21920725]

Svensson BG, Hallberg T, Nilsson A, Schütz A, Hagmar L. Parameters of immunological competence in subjects with high consumption of fish contaminated with persistent organochlorine compounds. Int Arch Occup Environ Health. 1994; 65:351–358. [PubMed: 8034358]

Taylor TR, Tucker T, Whalen MM. Persistent inhibition of human natural killer cell function by ziram and pentachlorophenol. Environmental Toxicology. 2005; 20:418–424. [PubMed: 16007644]

Thornton JW, McCally M, Houlihan J. Biomonitoring of industrial pollutants: Health and policy implications of the chemical body burden. Public Health Rep. 117:315–323.

Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol Ther. 2008; 117:244–79. [PubMed: 18155297]

Trejo-Acevedo A, Diaz-Barriga F, Carrizales L, Dominguez G, Costilla R, Ize-Lema I, Yarto-Ramirez M, Gavilan-Garcia A, Mejia-Saavedra JJ, Perez-Maldonado IN. Exposure assessment of persistent organic pollutants and metals in Mexican children. Chemosphere. 2009; 74:974–980. [PubMed: 19091374]

Tsukaguchi K, de Lange B, Boom WH. Differential Regulation of IFN-γ, TNF-α, and IL-10 Production by CD41 αβTCR1 T Cells and Vd21 γδ T Cells in Response to Monocytes Infected with *Mycobacterium tuberculosis*-H37Ra. Cellular Immunology. 1999; 194:12–20. [PubMed: 10357876]

Turusov V, Rakitsky V, Tomatis L. Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. Environmental Health Perspectives, Review. 2002; 110:125–128.

Udoji F, Martin T, Etherton R, Whalen MM. Immunosuppressive effects of triclosan, nonylphenol, and DDT on human natural killer cells, in vitro. J Immunotoxicology. 2010; 7:205–212.

Uhl S, Schmid P, Schlatter C. Pharmacokinetics of pentachlorophenol in man. Arch Toxicol. 1986; 58:182–186. [PubMed: 3964082]

U.S. Environmental Protection Agency (U.S. EPA). Restricted use product file. Oct 25.2001

Zaidi MR, Merlino G. The two faces of interferon-gamma in cancer. Clinical Cancer Research. 2011; 17:6118–6124. [PubMed: 21705455]

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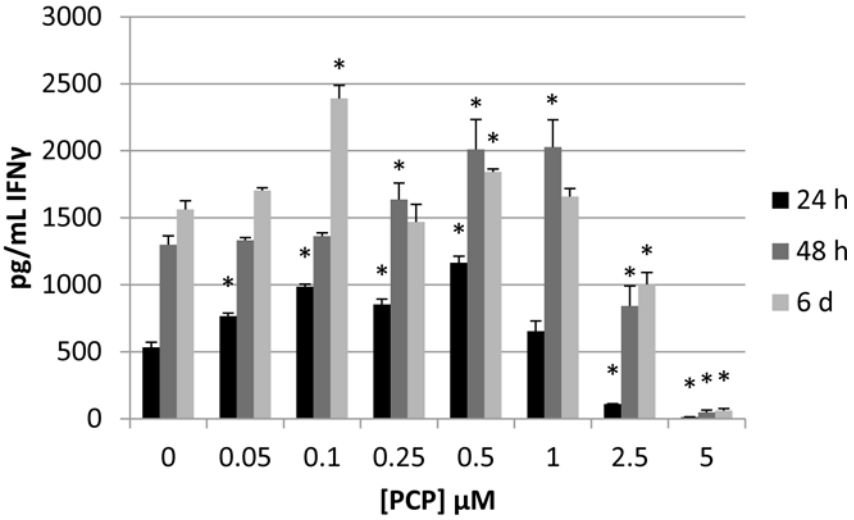

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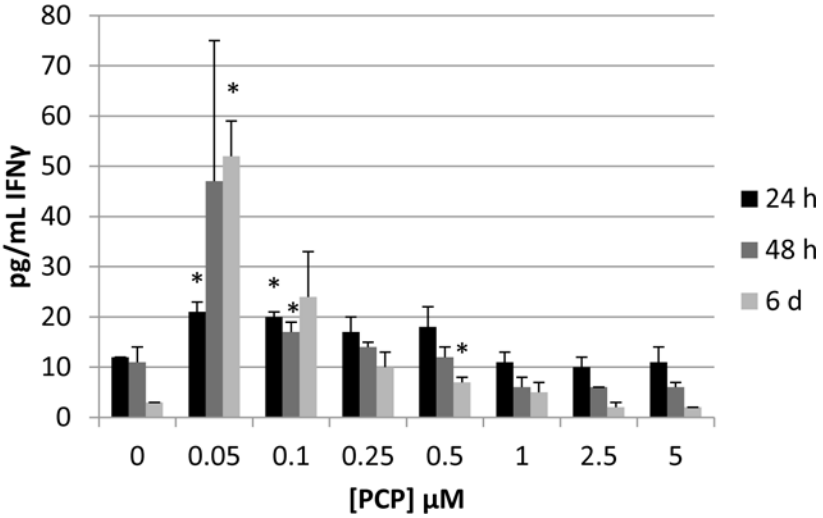

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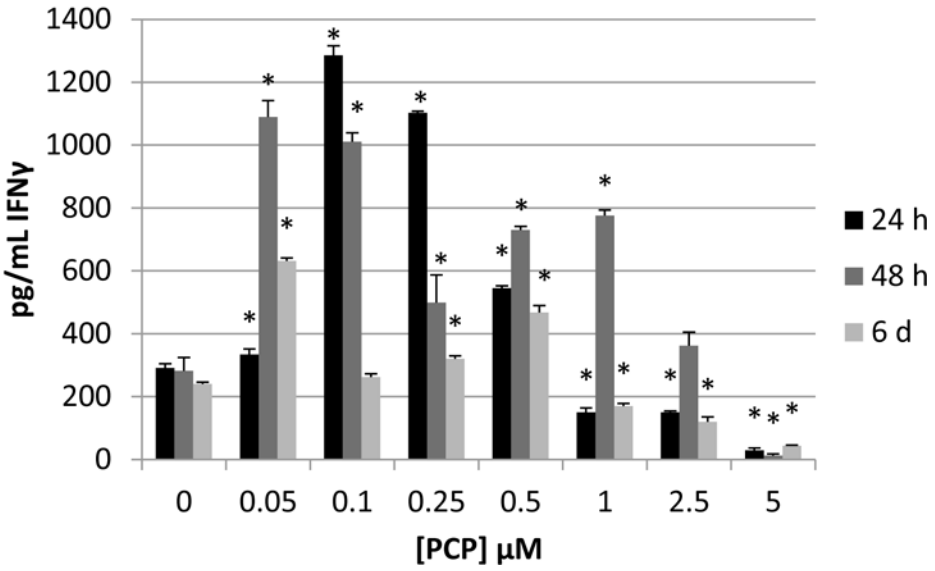

Figure 1.

Effects of 24 h, 48 h and 6 day exposures to PCP on IFNγ secretion from NK cells, MD-PBMCs, and PBMCs. A) NK cells exposed to 0.05–5µM PCP (cells from donor F365); B) MD-PBMCs exposed to 0.05–5µM PCP (cells from donor F312); C) PBMCs exposed to 0.05–5µM PCP (cells from donor F211). Values are mean±S.D. of triplicate determinations. * indicates a significant difference from the control ($p<0.05$)

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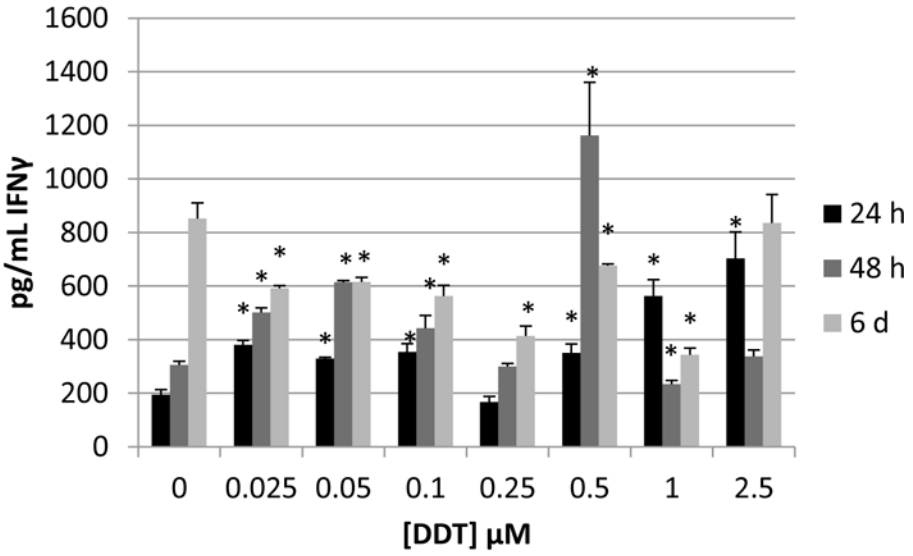

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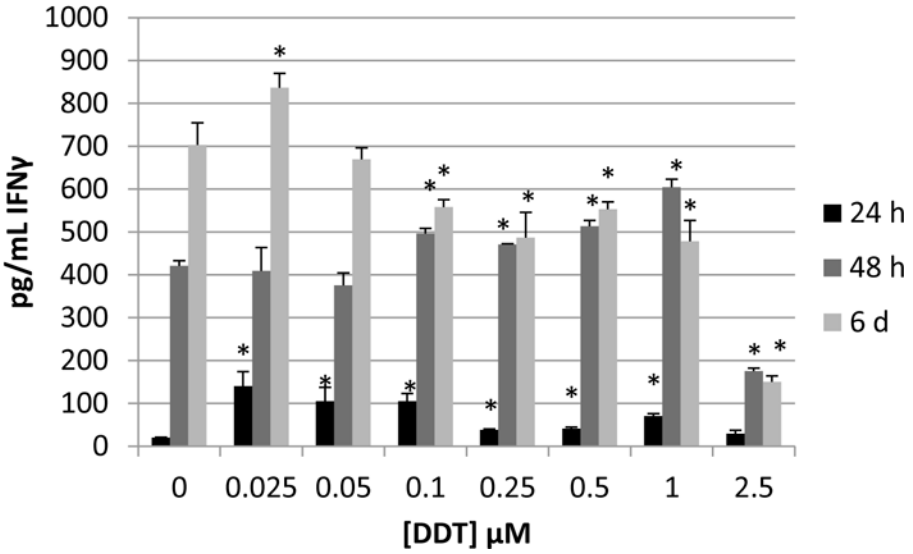

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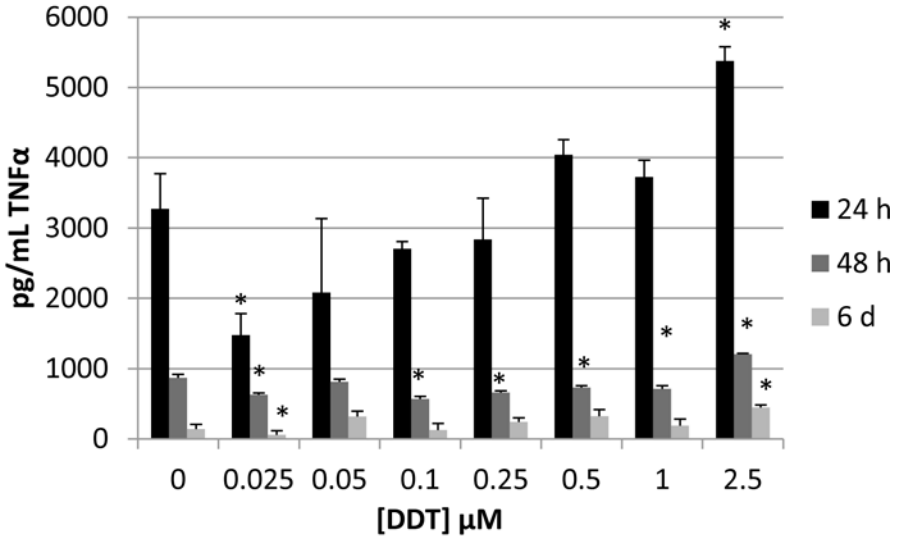

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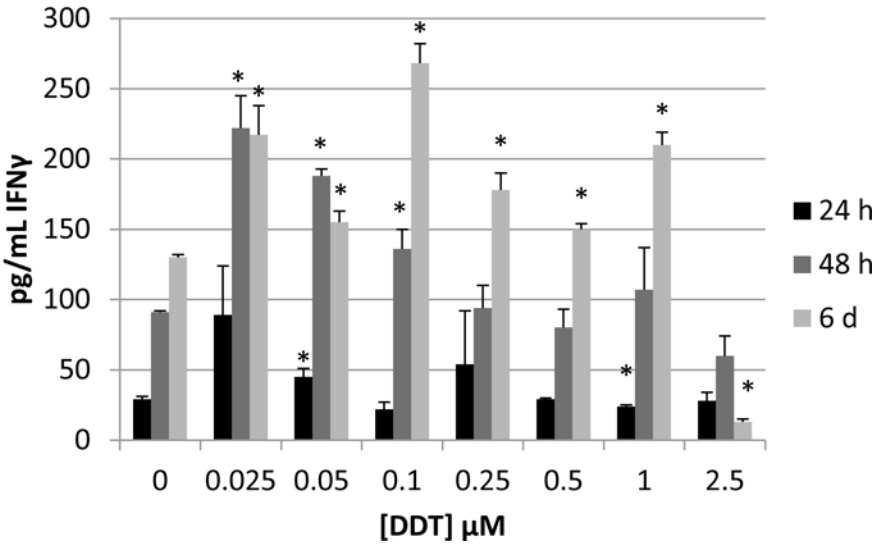

Figure 2.

Effects of 24 h, 48 h and 6 day exposures to DDT on IFNγ secretion from NK cells, MD-PBMCs, and PBMCs and TNFα secretion from MD-PBMCs. A) NK cells exposed to 0.025–2.5μM DDT (IFNγ secretion from cells of donor F366); B) MD-PBMCs exposed to 0.025–2.5μM DDT (IFNγ secretion from cells of donor F308); C) MD-PBMCs exposed to 0.025–2.5μM DDT (TNFα secretion from cells of donor F106); D) PBMCs exposed to 0.025–2.5μM DDT (IFNγ secretion from cells of donor F201). Values are mean±S.D. of triplicate determinations. * indicates a significant difference from the control ($p<0.05$)

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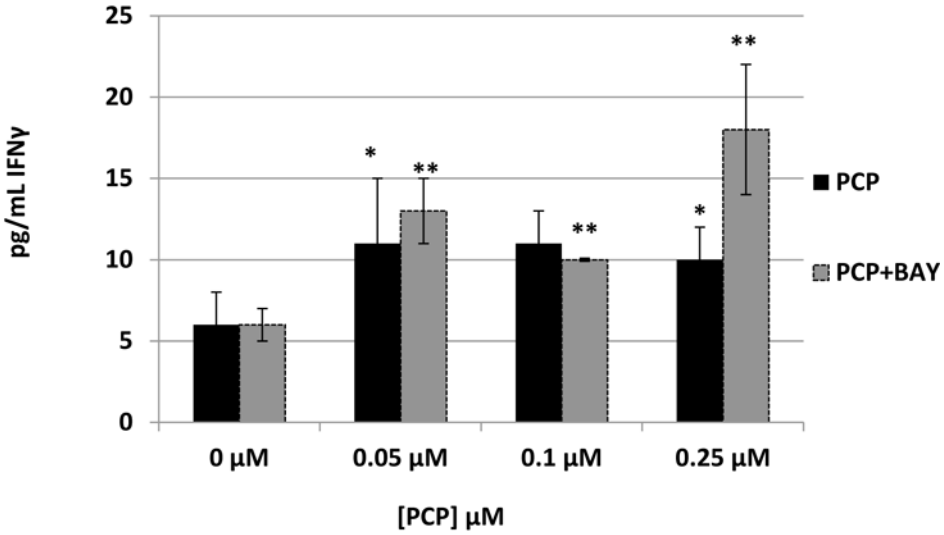

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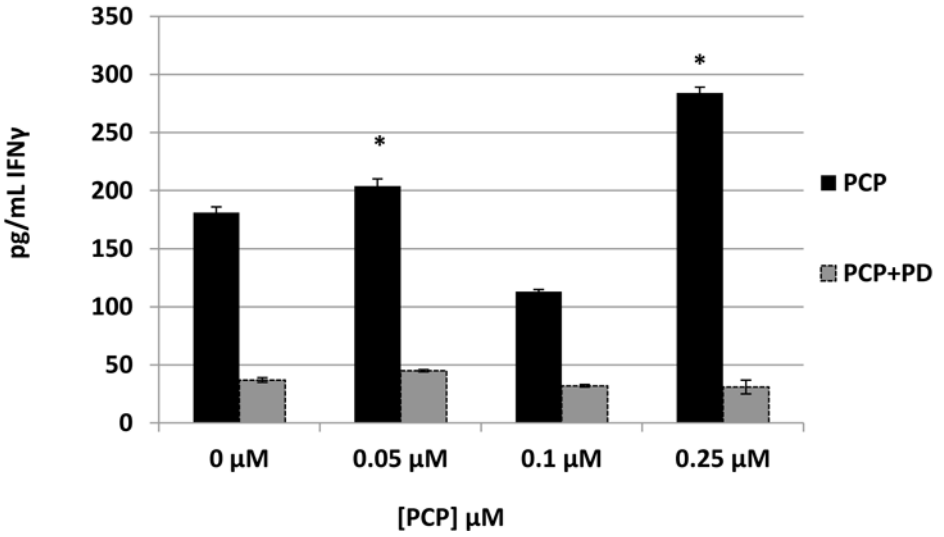

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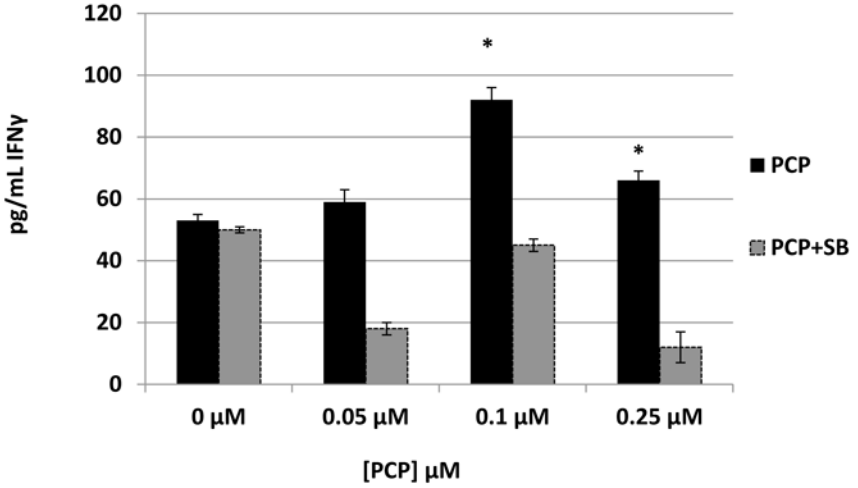

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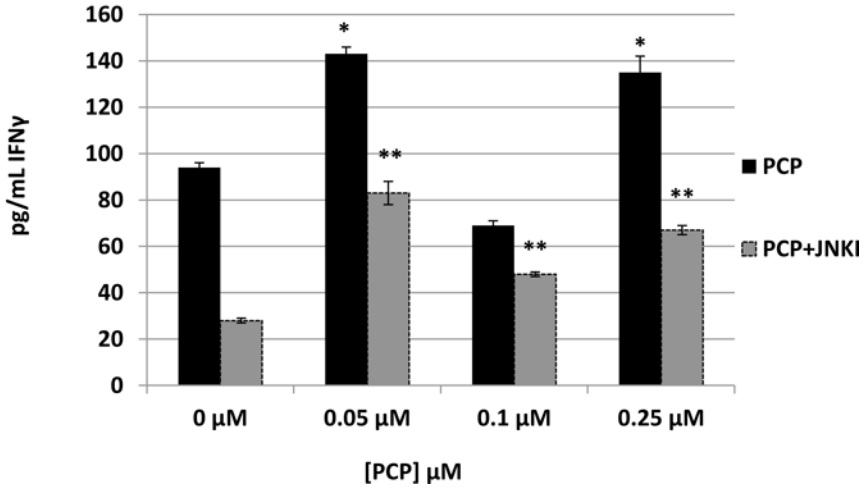

Figure 3.

Effects of 24 h exposure to 0.05, 0.1, and 0.25 μM PCP on IFNγ secretion from MD-PBMCs pre-treated with selective enzyme inhibitors. A) NFκB Inhibitor (BAY 11-7085) (cells from donor F374); B) MEK Inhibitor (PD98059) (cells from donor F326); C) p38 Inhibitor (SB202190) (cells from donor F331; D) JNK inhibitor (B178D3) (cells from donor F326). Values are mean±S.D. of triplicate determinations. * indicates a significant increase

compared to no PCP (0), $p<0.05$ **indicates a significant increase compared to no PCP + inhibitor, $p<0.05$.

Table 1A

Effects of 24 h, 48 h, 6 day exposures to PCP on IFNγ secretion from NK cells.

24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	**F335**	**F338**	**F345**	**F365**
0	1515±1	21±2	3400±50	532±39
0.05	29±1*	135±3*	2505±145≠	766±21*
0.1	31±0*	126±26*	5031±44*	984±20*
0.25	7±0≠	184±3*	3462±110	852±40*
0.5	69±1*	61±4*	1672±10≠	1163±50*
1	25±0*	32±1*	675±20≠	654±75
2.5	7±0≠	0±1≠	155±30≠	110±2≠
5	4±0≠	0±2≠	0±11≠	11±2≠
48 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	F335	F338	F345	F365
0	9±1	231±3	4623±352	1297±68
0.05	33±2*	94±2≠	5312±460	1331±21
0.1	123±2*	72±1≠	4969±72	1363±25
0.25	16±0.3*	111±2≠	3367±116≠	1636±123*
0.5	15±1*	739±5*	2331±19≠	2010±224*
1	10±0.2	177±12≠	873±89≠	2028±204*
2.5	3±1≠	53±3≠	200±56≠	841±150≠
5	0±0.7≠	8±1≠	0±15≠	47±17≠
6 day	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	F335	F338	F345	F365
0	87±0.7	228±5	5063±69	1563±64
0.05	48±2≠	142±39	2992±122≠	1702±21
0.1	100±1*	113±2≠	2806±26≠	2391±97*
0.25	105±1*	284±7*	4762±78≠	1469±130
0.5	17±2≠	100±5≠	1992±0≠	1842±22*
1	21±1≠	126±2≠	716±25≠	1658±60
2.5	8±0.4≠	14±2≠	503±69≠	1003±89≠
5	4±0.2≠	11±0.4≠	0±17≠	61±14≠

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 1B

Effects of 24 h, 48 h, 6 day exposures to PCP on TNFα secretion from NK cells.

24 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	**KB138**	**KB184**	**KB190**	**KB128**
0	170±12	742±11	72 ± 26	54±2
0.1	283±151	538±112	601±139 *	ND
0.25	97±17 ≠	681±31	562±32 *	51±7
0.5	106±9 ≠	687±17 ≠	344±101 *	35±1 ≠
1	215±88	816±46	390±183	38±3 ≠
2.5	0±23 ≠	456±21 ≠	176±80	12±1 ≠
5	0±0 ≠	295±31 ≠	238±74 *	0±1 ≠
48 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	KB138	KB184	KB190	KB128
0	56±14	66±178	0±2	407±20
0.1	68±15	0±19	0±2	ND
0.25	49±8	0±7	2±11	378±98
0.5	54±8	0±23	0±2	395±86
1	43±7	182±150	0±4	392±25
2.5	8±2 ≠	0±47	0±1	162±26 ≠
5	0±1 ≠	0±39	0±1	29±1 ≠
6day	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	KB138	KB184	KB190	KB128
0	21±4	0±12	0±25	244±36
0.05	21±2	0±7	0±16	ND
0.1	26±1	0±12	0±5	150±26 ≠
0.25	20±1	0±5	0±2	156±10 ≠
0.5	12±2 ≠	0±22	0±3	185±8
1	12±1 ≠	0±24	0±10	193±33
2.5	4±1 ≠	0±3	0±4	52±3 ≠
5	2±2 ≠	0±5	0±32	5±0 ≠

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 2A

Effects of 24 h, 48 h, 6 day exposures to PCP on IFNγ secretion from MD-PBMCs.

24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**				
[PCP] μM	**F209**	**F307**	**F308**	**F312**	**F314**
0	87±8	68±4	366±4	12±0	8±0
0.05	135±18 *	83±1 *	156±60 ≠	21±2 *	38±4 *
0.1	77±6	54±3 ≠	185±142	20±1 *	15±10
0.25	91±4	59±1	106±4 ≠	17±3	9±1
0.5	67±14	39±2 ≠	245±8 ≠	18±4	9±1
1	22±6 ≠	32±1 ≠	147±2 ≠	11±2	8±1
2.5	21±1 ≠	32±4 ≠	4±3 ≠	10±2	6±0 ≠
5	16±4 ≠	104±5 *	17±2 ≠	11±3	5±1 ≠
48 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**				
[PCP] μM	F209	F307	F308	F312	F314
0	298±7	86±25	236±8	11±3	6±1
0.05	348±93	97±32	443±28 *	47±28	124±20 *
0.1	377±33 *	39±25	856±173 *	17±2 *	43±22
0.25	266±7 ≠	57±11	351±11 *	14±0	45±17
0.5	263±11 ≠	49±25	247±13	12±2	8±1
1	75±14 ≠	24±6 ≠	743±26 *	6±2	6±1
2.5	18±17 ≠	33±3	176±11 ≠	6±0	5±1 ≠
5	5±1 ≠	188±31 *	243±6	6±1	6±1 ≠
6day	**Interferon gamma secreted in pg/mL (mean±S.D.)**				
[PCP] μM	F209	F307	F308	F312	F314
0	210±31	153±5	161±1	3±0	1±0.2
0.05	295±118	174±16	641±163 *	52±7 *	81±36
0.1	215±17	246±13 *	216±54	24±9	9±5

24 h	Interferon gamma secreted in pg/mL (mean±S.D.)				
[PCP] μM	F209	F307	F308	F312	F314
0.25	218±2	197±7*	205±13*	10±3	7±2*
0.5	153±37	220±19*	390±17*	7±1*	2±1
1	135±11≠*	210±2*	304±10*	5±2	1±0.4
2.5	99±49≠	217±9*	130±1≠	2±1	1±0.4
5	31±4≠	445±4*	0±7≠	2±0	0.3±1

Values are mean±S.D. of triplicate determinations.

*Indicates a significant increase

≠indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 2B

Effects of 24 h, 48 h, 6 day exposures to PCP on TNFα secretion from MD-PBMCs.

24 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	**F100**	**F101**	**F106**	**F170**
0	389±23	777±27	1767±133	381±16
0.1	ND	432±357	1418±175	455±108
0.25	450±98	650±10≠	1550±78	252±32
0.5	373±8	572±40≠	1235±35≠	397±78
1	471±123	648±12≠	1863±232	399±36
2.5	492±62	671±82	1490±113	143±27≠
5	450±32	829±13	1470±182	476±204
10	405±95	776±24	857±30≠	587±41
48 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	F100	F101	F106	F170
0	192±19	239±4	834±42	3±40
0.1	ND	296±135	797±44	64±8
0.25	249±46	459±105	942±446	89±17
0.5	227±42	301±95	1010±10*	90±20
1	305±18*	340±24*	10950±8357	98±15.0
2.5	330±34*	257±4*	824±16	117±9*
5	269±13*	317±3*	957±74	187±11*
10	203±41	372±4*	717±234	219±5*
6day	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	F100	F101	F106	F170
0	524±22	100±11	1072±72	301±15
0.05	ND	54±16≠	573±82≠	215±42
0.1	358±32≠	47±6≠	129±19≠	239±37
0.25	287±45≠	25±12≠	653±34≠	230±28≠
0.5	183±69≠	25±27≠	888±38≠	249±47
1	169±61≠	0±3≠	656±30≠	254±40
2.5	169±61≠	0±5≠	670±10≠	250±14≠
5	132±34≠	0±6≠	470±10≠	250±25

Values are mean±S.D. of triplicate determinations.

*Indicates a significant increase

≠indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 3A

Effects of 24 h, 48 h, 6 day exposures to PCP on IFNγ secretion from PBMCs

24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	**F197**	**F211**	**F214**	**F238**
0	19±2	291±14	92±5	17±2
0.05	46±27	334±18 *	462±56 *	16±6
0.1	44±4 *	1286±30 *	206±145	20±8
0.25	20±3	1103±5 *	128±46	22±3
0.5	13±6	545±7 *	68±6 ≠	13±3
1	7±2 ≠	150±14 ≠	59±3 ≠	21±2
2.5	8±2 ≠	150±5 ≠	64±16	22±4
5	8±2 ≠	29±7 ≠	35±16 ≠	61±2
48 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	F197	F211	F214	F238
0	27±2	282±43	253±8	94±13
0.05	50±18	1089±52 *	497±22 *	100±2
0.1	37±4 *	1011±28 *	355±52	101±1
0.25	33±5	499±88 *	238±122	100±1
0.5	20±1 ≠	730±11 *	263±140	67±8 ≠
1	12±4 ≠	776±18 *	208±22	71±2
2.5	15±5 ≠	362±43	68±5 ≠	63±4 ≠
5	5±1 ≠	12±6 ≠	16±11 ≠	86±3
6day	**Interferon gamma secreted in pg/mL (mean±S.D.)**			
[PCP] μM	F197	F211	F214	F238
0	43±13	240±6	70±3	65±2
0.05	218±165	632±9 *	144±2 *	79±2 *
0.1	192±66	262±11	57±25	76±5
0.25	107±8 *	321±9 *	63±11	103±3 *
0.5	4±7 ≠	468±22 *	62±8	84±3 *
1	26±9	170±8 ≠	80±24	82±6 *
2.5	55±11	120±16 ≠	58±15	47±3 ≠
5	5±9 ≠	44±2 ≠	29±3 ≠	80±2 *

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 3B

Effects of 24 h, 48 h, 6 day exposures to PCP on TNFα secretion from PBMCs.

24 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	**F121**	**F123**	**F146**	**F151**
0	722±27	206±6	1303±114	1737±30
0.1	633±60	182±7≠	1276±103	764±117≠
0.25	659±40	190±5≠	1154±49	728±53≠
0.5	655±38	188±14	1257±134	948±81≠
1	725±20	208±15	1162±75	1210±88≠
2.5	728±22	256±12*	1091±54	1235±181≠
5	779±13*	426±24*	1488±104	999±81≠
48 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	F121	F123	F146	F151
0	81±9	37±4	616±13	680±44
0.1	130±23*	49±1*	884±74*	318±41≠
0.25	44±15≠	41±2	560±34	441±58≠
0.5	14±7≠	43±1	797±61*	487±52≠
1	18±15≠	51±2*	780±32*	380±34≠
2.5	39±12≠	71±6*	1094±32*	499±34≠
5	67±4	131±15*	514±57	218±31≠
6 day	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[PCP] uM	F121	F123	F146	F151
0	450±11	88±2	637±87	975±.85
0.05	512±4*	54±2≠	322±155	694±59≠
0.1	494±16*	68±4≠	452±154	777±106
0.25	476±11*	53±2≠	346±177	743±178
0.5	462±51	69±1≠	509±64	909±57
1	499±12*	62±3≠	516±176	924±94
2.5	484±13*	58±5≠	314±122≠	840±41
5	462±6	46±2≠	341±35≠	662±121≠

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 4A

Effects of 24 h, 48 h, 6 day exposures to DDT on IFNγ secretion from NK cells.

24 h	Interferon gamma secreted in pg/mL (mean±S.D.)			
[DDT] μM	F351	F361	F366	F375
0	6973±107	32±4	195±18	88±6
0.025	10003±140*	26±5	381±16*	66±8≠
0.05	11156±201*	30±5	329±5*	83±6
0.1	9713±483*	31±2	354±32*	98±4
0.25	8912±517*	20±3≠	167±21	117±9*
0.5	7705±266*	22±0	351±33*	104±3*
1	10675±283*	17±1≠	563±61*	133±3*
2.5	1997±60≠	32±4	703±99*	101±3*
48 h	Interferon gamma secreted in pg/mL (mean±S.D.)			
[DDT] μM	F351	F361	F366	F375
0	24086±368	24±2	305±15	152±6
0.025	19420±1140≠	175±30*	501±18*	130±26
0.05	19172±1402≠	50±40	615±6*	84±8≠
0.1	26496±1038*	26±24	443±47*	221±20*
0.25	27048±1321	10±4≠	300±11	116±4≠
0.5	30763±1029*	6±1≠	1163±198*	98±2≠
1	18963±15299	3±1≠	234±14≠	142±7
2.5	20439±243≠	5±1≠	338±23	132±11
6day	Interferon gamma secreted in pg/mL (mean±S.D.)			
[DDT] μM	F351	F361	F366	F375
0	14108±782	13±6	852±59	66±4
0.025	23330±1034*	163±28*	592±10≠	146±120
0.05	25531±261*	46±37	616±17≠	66±10
0.1	13142±189	24±22	563±40≠	91±15
0.25	19323±737*	9±4	414±37≠	66±3
0.5	13038±695	6±1	677±6≠	69±3
1	21802±762*	3±1	344±25≠	55±1≠
2.5	6705±1144≠	4±0	836±106	56±6

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 4B

Effects of 24 h, 48 h, 6 day exposures to DDT on TNFα secretion from NK cells.

24 h	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] M	KB171	KB178	KB185	KB190
0	33±1	50±18	0±1	6±8
0.025	36±2	0±43	0±2	4±22
0.05	36±2	36±39	0±2	41±17
0.1	35±1	0±7ǂ	0±3	46±41
0.25	67±54	74±36	0±1	15±1
0.5	38±2	39±49	1±3	18±10
1	72±55	0±19ǂ*	0±3	26±16
2.5	35±2	53±54	7±4*	7±1

48 h	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] μM	KB171	KB178	KB185	KB190
0	4±2	221±7	6±2	17±6
0.025	7±3	277±43	8±1	11±5
0.05	5±2	322±24*	7±1	7±5
0.1	7±1	295±20*	8±1	8±4
0.25	7±3	346±31*	9±1	28±12
0.5	7±1	306±25*	9±1	14±2
1	6±2	451±42*	8±1	20±16
2.5	6±1	342±63	8±1	13±2

6 day	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] μM	KB171	KB178	KB185	KB190
0	0±1	3±7	2±1	1±1
0.025	0±1	2±3	1±1	1±1
0.05	0±2	1±1	2±1	0±2
0.1	0±3	6±2	2±2	1±1
0.25	0±1	1±3	1±1	2±1
0.5	0±1	9±7	1±1	0±5
1	0±1	3±2	7±6	3±2
2.5	0±1	5±5	2±1	2±1

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

ǂ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 5A

Effects of 24 h, 48 h, 6 day exposures to DDT on IFNγ secretion from MD-PBMCs.

24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	**F303**	**F304**	**F307**	**F308**	**F312**	**F314**
0	5±1	20±6	153±26	20±1	10±1	13±2
0.025	18±6	12±4	153±13	140±34*	11±3	25±9
0.05	9±2	24±13	132±5	105±32*	11±1	14±1
0.1	8±1*	34±23	123±16	105±18*	12±1	14±1
0.25	5±1	15±2	106±8	38±2*	9±2	14±1
0.5	5±0.3	20±1	100±19†*	41±4*	14±4	13±2
1	7±1*	19±2	133±11	70±6*	11±0.3	12±1
2.5	5±2	34±1	331±16*	29±8	7±1≠	13±1
48 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	F303	F304	F307	F308	F312	F314
0	6±0.4	4±1	222±15	421±12	7±0.3	17±2
0.025	22±8	134±80	172±4≠	409±54	22±5*	21±1*
0.05	12±1*	35±10*	168±13≠	375±29	16±3*	21±1
0.1	15±4	24±1*	315±2*	496±12*	12±1*	22±1*
0.25	10±0.2*	16±23	204±13	471±0*	9±0.3*	24±1*
0.5	6±0.4	6±1	127±10≠	513±14*	9±0.3*	19±3
1	9±4	9±0.3*	228±23	604±19*	8±1	17±2
2.5	7±0.07	17±1*	531±14*	175±7≠	7±0.3	21±4
6day	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	F303	F304	F307	F308	F312	F314
0	51±11	28±2	430±11	703±51	5±0.3	18±1
0.025	97±13*	28±16	292±18≠	836±34*	9±3	24±2*
0.05	46±3	27±5	258±5≠	669±27	5±0.05	22±1*

24 h	Interferon gamma secreted in pg/mL (mean±S.D.)					
[DDT] μM	**F303**	**F304**	**F307**	**F308**	**F312**	**F314**
0.1	55±11	30±5	324±14$^{\neq}$	558±17$^{\neq}$	5±1	23±1*
0.25	67±5	24±4	260±12$^{\neq}$	486±59$^{\neq}$	5±0.4	24±2*
0.5	47±2	15±3$^{\neq}$	193±12$^{\neq}$	553±17$^{\neq}$	5±0.1	22±1*
1	25±2$^{\neq}$	41±15	385±14$^{\neq}$	478±49$^{\neq}$	6±0.1	26±1*
2.5	19±4$^{\neq}$	35±8	476±12*	150±14$^{\neq}$	4±0.2	18±2

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

$^{\neq}$ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 5B

Effects of 24 h, 48 h, 6 day exposures to DDT on TNFα secretion from MD-PBMCs.

24 h	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] uM	F106	F174	F178	F180
0	3273±498	631±35	1605±112	712±20
0.025	1478±301ǂ	461±61ǂ	931±141ǂ	444±73ǂ
0.05	2083±1051	455±17ǂ	1544±48	400±9ǂ
0.1	2704±102	452±19ǂ	1403±86	392±28ǂ
0.25	2837±585	406±4ǂ	1492±64	402±39ǂ
0.5	4042±216	454±38ǂ	1518±111	409±43ǂ
1	3724±242	525±88	1352±119	476±82
2.5	5375±200*	570±31	2142±9*	1015±45*

48 h	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] uM	F106	F174	F178	F180
0	869±49	778±22	526±57	0±35
0.025	627±24ǂ	454±0ǂ	250±54ǂ	0±14
0.05	808±40	497±0ǂ	278±53ǂ	0±35
0.1	568±35ǂ	530±22ǂ	300±22ǂ	0±52
0.25	659±26ǂ	702±16ǂ	378±34ǂ	0±36
0.5	731±23ǂ	683±14ǂ	454±45	0±15
1	712±42ǂ	688±58	573±107	0±35
2.5	1205±12*	1388±64*	740±66*	0±10

6day	TNF alpha secreted in pg/mL (mean±S.D.)			
[DDT] uM	F106	F174	F178	F180
0	140±65	784±41	659±35	171±19
0.025	57±61ǂ	317±37ǂ	12±48ǂ	218±191
0.05	319±76	429±22ǂ	214±143ǂ	101±10ǂ
0.1	124±93	381±54ǂ	132±31ǂ	94±4ǂ
0.25	243±58	431±12ǂ	196±56ǂ	126±29
0.5	321±96	465±98ǂ	148±4ǂ	654±39*
1	186±96	555±186	176±74ǂ	88±11ǂ
2.5	445±36*	641±8ǂ	379±18ǂ	197±5

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

ǂ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 6A

Effects of 24 h, 48 h, 6 day exposures to DDT on IFNγ secretion from PBMCs.

24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	**F109**	**F111**	**F113**	**F201**	**F220**	**F229**
0	399±34	297±9	154±20	29±2	336±2	340±8
0.025	566±22 *	683±9 *	120±23	89±35	425±12 *	195±14 ≠
0.05	360±25	646±34 *	131±20	45±6 *	426±4 *	107±6 ≠
0.1	396±14	673±35 *	109±6 ≠	22±5	500±38 *	116±2 ≠
0.25	364±25	611±23 *	112±1	54±38	344±7	84±4 ≠
0.5	376±15	624±14 *	96±7 ≠	29±1	252±2 ≠	134±6 ≠
1	442±16	457±30 *	110±3	24±1	230±5 ≠	26±6 ≠
2.5	582±106	280±5	123±14	28±6	122±6 ≠	96±1 ≠
48 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	F109	F111	F113	F201	F220	F229
0	858±25	493±33	137±9	91±1	618±15	217±3
0.025	209±13	1615±32 *	115±18	222±23 *	846±27 *	285±9 *
0.05	825±30	1832±46 *	81±8 ≠	188±5 *	653±32	183±12 ≠
0.1	1013±32 *	1632±34 *	122±23	136±14 *	609±29	284±9 *
0.25	1096±16 *	1167±40 *	140±15	94±16	528±35 ≠	262±8 *
0.5	968±20 *	1214±9 *	114±9 ≠	80±13	528±28 ≠	74±3 ≠
1	1072±31 *	1024±8 *	110±20	107±30	480±23 ≠	200±5 ≠
2.5	1184±23 *	730±16 *	116±9 ≠	60±14	130±4 ≠	78±5 ≠
6 day	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[DDT] μM	F109	F111	F113	F201	F220	F229
0	770±58	5656±104	87±4	130±2	1406±12	166±4
0.025	460±64 ≠	22097±3952 *	100±4 *	217±21 *	896±33 ≠	94±10 ≠
0.05	1098±65 *	15419±85 *	73±5 ≠	155±8 *	838±37 ≠	174±11

24 h	Interferon gamma secreted in pg/mL (mean±S.D.)					
[DDT] μM	F109	F111	F113	F201	F220	F229
0.1	0±12*	14268±3738	74±5≠	268±14*	831±9≠	189±2*
0.25	598±45≠	9903±469*	84±9	178±12*	984±40≠	270±7*
0.5	668±26	8280±242*	91±6	150±4*	1177±9≠	203±4*
1	602±30≠	7215±194*	98±4*	210±9*	928±10≠	246±5*
2.5	694±36	4602±81≠	80±4	13±2≠	220±3≠	73±8≠

Values are mean±S.D. of triplicate determinations.

*Indicates a significant increase

≠indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 6B

Effects of 24 h, 48 h, 6 day exposures to DDT on TNFα secretion from PBMCs.

24 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[DDT] uM	**F110**	**F111**	**F112**	**F164**
0	441±29	2837±95	2077±76	8±10
0.025	429±109	2859±107	1941±39	62±28
0.05	460±100	2820±123	1860±77≠	83±7*
0.1	380±5	3080±94*	1843±52≠	92±22*
0.25	431±15	3237±29*	2057±62	211±12*
0.5	410±36	3037±91	1897±37≠	177±32*
1	428±17	3093±46*	2007±58	362±23*
2.5	591±34*	3219±98*	2195±24	581±65*
48 h	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[DDT] uM	F110	F111	F112	F164
0	74±2	1945±166	671±16	51±4
0.025	77±1	2087±190	681±91	178±42*
0.05	77±5	2054±354	638±5	157±37*
0.1	67±1≠	2012±272	636±18	120±19*
0.25	79±7	2326±4	684±20	142±13*
0.5	92±7*	2412±75*	632±10≠	166±15*
1	100±4*	1982±306	694±22	163±22*
2.5	134±8*	2140±67	638±169	282±9*
6 day	**TNF alpha secreted in pg/mL (mean±S.D.)**			
[DDT] uM	F110	F111	F112	F164
0	9±1	1905±571	27±9	82±1
0.025	9±1	14063±650	19±8	330±266
0.05	5±1≠	14682±35	23±2	144±74
0.1	7±1	1375±126	23±3	234±125
0.25	9±1	1510±64	22±1	124±24
0.5	9±1	1489±22	27±2	234±122
1	9±2	1485±74	25±3	173±57
2.5	13±2	1770±60	28±9	369±67*

Values are mean±S.D. of triplicate determinations.

* Indicates a significant increase

≠ indicates a significant decrease in secretion compared to control cells (cells treated with vehicle alone), $p<0.05$

ND=Not Determined

Table 7

Effects of 24 h exposure to PCP in the presence and absence of specific signaling pathway inhibitors on IFNγ secretion from MD-PBMCs

NFκB inhibitor (BAY 11-7085)						
24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**					
[PCP] μM	**F331**	**F358**	**F360**	**F374**	**F389**	**F390**
0	119±4	12±0.1	7±0.3	6±2	179±23	644±30
0 + B	104±3	8±0.3	6±1	6±1	156±7	600±49
0.05	149±4 *	14±1 *	7±1	11±4	198±3	793±43 *
0.05+B	131±2 **	21±12	6±1	13±2 **	121±17	1100±82 **
0.1	125±4	16±1 *	7±1	11±2 *	150±28	815±48 *
0.1+ B	124±3 **	32±19	6±1	10±0.1 ***	124±25	802±55 **
0.25	138±4 *	20±3 *	9±0 *	10±2 *	227±14 *	826±86 *
0.25+B	110±13	70±10 **	5±1	18±4 **	231±7 **	570±16

ERK1/2 pathway inhibitor (PD98059)								
24 h	**Interferon gamma secreted in pg/mL (mean±S.D.)**							
[PCP] μM	**F326**	**F331**	**F358**	**F359**	**F360**	**F 370**	**F385**	**F389**
0	181±5	98±2	29±2	10±0.3	6±2	14±1	187±50	200±0.3
0 + PD	37±2	38±1	19±1	7±0.3	4±1	9±1	47±10	72±9
0.05	204±6 *	121±4 *	37±1 *	12±1	7±1	18±5	255±26	304±6 *
0.05+PD	45±1 **	39±1	27±2 **	11±2	10±2 **	13±3	115±4 **	102±17
0.1	113±2	116±4 *	36±4 *	11±1	8±0.4	18±5	287±42	201±2
0.1+ PD	32±1	38±1	20±0.3	8±2	6±1	22±6	65±3	69±5
0.25	284±5 *	92±3	43±1 *	14±1 *	12±2 *	21±3 *	291±28 *	204±15
0.25+PD	31±6	37±2	14±1	1±1	10±4	53±6 **	69±14 **	63±4